Monkey Brain Arylamidase: Studies on Characteristics and Mode of Hydrolysis of Physiologically Active Peptides(Dissertation_全文)

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学位申請論文

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論　文　目　錄

主論文

1. 題　目

Monkey Brain Arylamidase. — Studies on Characteristics and Mode of Hydrolysis of Physiologically Active Peptides.

（サル脳アリルアミダーゼ．－性質と生理活性ペプチド分解機作に関する研究）

2. 公表の方法．Journal of Biochemistry　投稿中

3. 冊数　　1冊

参考論文

1. 題　目

Purification and Properties of Oxytocinase (Cystine Aminopeptidase) from Monkey Placenta.

（サル胎盤のオキシトチナーゼ（シスチンアミノペプチダーゼ）の精製と性質）

Journal of Biochemistry Vol. 80, No.2 pp 389-396 (1976) 掲載　大島清と共著

2. 題　目

Purification and Characterization of Arylamidase from Monkey Brain.

（サル脳よりアリルアミダーゼの精製と性質）

Journal of Biochemistry Vol. 81, No.3 pp. 631-639 (1977) 掲載　大島清と共著

昭和53年6月28日

学位授与申請者

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論文内容の要旨

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論文題目

Monkey Brain Arylamidase. — Studies on Characteristics and Mode of Hydrolysis of Physiologically Active Peptides.

(サル脳アリルアミダーゼ一性質と生理活性ペプチド分解機作に関する研究)

哺乳類脳組織には.種々のアミノ酸β-ナフチルアミドヌはp-ニトロアニリドを水解する酵素，アリルアミダーゼの存在が知られておりラット，牛大脳の酵素についてその酵素化学的性質が一部明らかにされている。一方本酵素の生理学的意義について，脳内各種ペプチド類分解代謝に関与する可能性が指摘されている。

近年筆者等はL-アラニンβ-ナフチルアミドを基質として使用し，サル大脳より本酵素の精製法を確立しその酵素化学的性質を一部明らかにした。本論文では酵素の大量精製を試

み更にその酵素化学的性質を明らかにすると共に各種生理活性ペプチド類への作用機作について検討し以下の結果を得た。

本酵素のアミノ酸分析の結果，酸性アミノ酸に富む酸性タンパク質であり　システインが少なく，又アラニン，バリン　ロイシン等のアミノ酸の多い点が特徴的であった。ダンシル法で同定したアミノ末端基はアラニンであった。酵素活性はアミノペプチダーゼの特異的阻害剤(ベスタチン)により拮抗阻害されKi値は$2.5\times10^{-7}M$とピュロマイシンに対するKi値($1.5\times10^{-7}M$)と同程度であった。酵素はスルフヒドリル試薬非存在下で不活性化され，β-メルカプトエタノールで100%活性を回復，一方ジチオスレイトールで約2.5倍の活性化を受けた。本酵素のアミノペプチダーゼ活性を近年報告されているL-アミノ酸オキシダーゼ法で測定した。その結果，L-ロイシル-グリシン，L-ロイシル-グリシル-グリシン等の合成ペプチドを水解する事　又各種生理活性ペプチド類では　N

末端に中性アミノ酸を有するエンケファリン類，メチオニルーリジルーブラジキニン等を最もよく水解し，アンギオテンシンI,II，MI戸等も水解するがN末端にピログルタミン酸をもつLH-RH，TRH，N末端の次にプロリンをもつP物質，ブラジキニンは水解しない事が明らかとなった。又アミノペプチダーゼ活性はピュロマイシン，ベスタチンにより拮抗的に阻害された。ペプチド類に対するKm値を比較すると長い側鎖をもつペプチドに対して低い値をもつ傾向が見られた。

ペプチド類の分解産物をダンシル誘導体として同定した結果　本酵素はN末端より順次水解するエキソペプチダーゼである事，アンギオテンシナーゼ活性及びキニン変換活性をもち，エンケファリン類，MI戸等N末端より水解する性質をもつ事が明らかとなった。

以上の結果，本酵素は脳における種々の生理活性ペプチド類の放出，又は分解代謝に関与する可能性があり　各種阻害剤は　これ等ペ

プチド類の脳内代謝を明らかにする上で有効であろうとの結論を得た。

Monkey Brain Arylamidase.

- Studies on Characteristics and Mode of Hydrolysis of Physiologically Active Peptides.

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Abbreviations: LH-RH, luteinizing hormone-releasing hormone; TRH, thyrotropin-releasing hormone; MIF, melanocyte-stimulating hormone release-inhibitory factor; bestatin, [(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine; SDS, sodium dodecyl sulfate.

SUMMARY

An extensive purification of monkey brain arylamidase was carried out. The amino acid analyses indicate that the enzyme is quite rich in acidic amino acid and is poor in cystine. The amino terminal residue was determined to be alanine by the dansylation method. The enzyme was activated with sulfhydryl compounds. Dithiothreitol was more effective than β-mercaptoethanol. Bestatin competitively inhibited the enzyme activity and the Ki value was calculated to be 2.5×10^{-7}M, which was of the same order as that of puromycin. The inhibitions by puromycin and bestatin were reversible. The enzyme hydrolyzed di-, tri- and oligopeptides including physiologically active peptides. Among various physiologically active peptides, enkephalins and Met-Lys-bradykinin which possess a neutral amino acid at the N-terminal position were more rapidly hydrolyzed by the enzyme. The peptides such as LH-RH and TRH which possess a pyrrolidonecarboxylyl group at the N-terminal position and substance P and bradykinin which possess a proline residue adjacent to the N-terminal residue were not hydrolyzed by the enzyme. The Km values for various peptides indicate that the enzyme has higher affinity for oligopeptides than di- and tripeptides. The aminopeptidase activity of the enzyme was also competitively inhibited by puromycin and bestatin. The analyses of the hydrolysis products of various peptides by the dansylation method indicate that the enzyme has both kinin-converting activity and angiotensinase activity.

INTRODUCTION

In various mammalian brain tissues, there exist enzymes which hydrolyze various amino acid β-naphthylamides or p-nitroanilides (1-3). These enzymes have been termed arylamidase after the suggestion by Patterson et al.(4) and their subgroups have been distinguished (5,6) : a) arylamidases with a preference for basic amino acid β-naphthylamide or p-nitroanilide (aminopeptidase B) ; b) arylamidases with a preference for acidic amino acid β-naphthylamide or p-nitroanilide (aminopeptidase A) ; c) arylamidases with a broad specificity but a preference for L-alanine and L-leucine β-naphthylamide or p-nitroanilide.

From rat brain, group a) and c) arylamidases have been partially purified and characterized (1). The first purification and characterization of group c) enzyme have been accomplished by Brecher et al. using bovine brain (2,3). A marked inhibition by a low concentration of puromycin and dependence of the activity on thiol groups are general characteristics of these enzymes.

The physiological functions of these enzymes have not yet been elucidated. However, their possible roles in the release or degradation of some physiologically active peptides have been suggested in the literatures (7-9).

In the previous paper (10), the purification and some characterization of group c) arylamidase from monkey brain have been reported. The enzyme hydrolyzed various amino acid β-naphthylamides, among which L-alanine β-naphthylamide was hydrolyzed most rapidly. Thiol groups were involved in the active site of the enzyme and puromycin was a competitive inhibitor for the enzyme (Ki : 5×10^{-7}M). The molecular weight of the enzyme was 92,000.

Recently, Umezawa et al. (11-13) have purified and characterized a specific aminopeptidase inhibitor (bestatin) from actinomycetes. This showed a strong inhibition on leucine aminopeptidase of swine kidney and aminopeptidase B of rat liver but did not show any inhibition on aminopeptidase A, trypsin, chymotrypsin, elastase, papain and thermolysin.

The present paper describes an extensive purification of a group c) arylamidase from monkey brain and its further characteristics, amino acid composition, N-terminal amino acid, inhibition by bestatin and effects of sulfhydryl compounds. The hydrolysis of various peptides including physiologically active peptides, Km values and their degradation mechanisms are also described. A preliminary report has appeared elsewhere (14).

EXPERIMENTAL

Materials – Japanese monkeys (Macaca fuscata fuscata) were anesthetized with ketamine hydrochloride and killed by exanguination. The brains were immediately removed and stored at -20°C until use. L-Alanine β-naphthylamide, Fast Garnet GBC salt (o-aminoazotoluenediazonium salt), L-alanyl-alanine, L-alanyl-alanyl-alanine, L-alanyl-proline, L-glycyl-glycine, L-phenylalanyl-sarcosine, L-amino acid oxidase (Type I), horseradish peroxidase (Type II) and o-dianisidine (3,3'-dimethoxy benzidine) were obtained from Sigma Chemical Co., Saint Louis, Missouri, U.S.A. L-Leucyl-glycyl-glycine, L-leucyl-glycine, carbobenzoxy-L-phenylalanyl-tyrosine, bradykinin, Lys-bradykinin, Met-Lys-bradykinin, Leu^5-enkephalin, Met^5-enkephalin, Ile^5-angiotensin I, II, substance P, luteinizing hormone-releasing hormone(LH-RH), thyrotropin-releasing hormone (TRH), and melanocyte-stimulating hormone release-inhibitory factor (MIF) were purchased from Protein Research Foundation, Osaka, Japan. Bestatin was kindly supplied by Dr. H. Umezawa. Puromycin and dansyl chloride were products of Makor Chemicals Ltd., Jerusalem, Israel and Pierce Chemical Co., Illinois, U.S.A., respectively. Diethylaminoethyl cellulose (DE-32) and hydroxylapatite were products of Whatman Biochemical Ltd., England and Seikagaku Kogyo Co., Tokyo, Japan, respectively. Sephadex G-25 and G-200 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Polyamide layer sheets were products of Cheng Chin Trading Co.Ltd., Taipei, Taiwan. All other chemicals were of reagent grade. L-Pyrrolidonyl β-naphthylamide was synthesized according to the method of Szewczuk et al. (15).

Determination of Protein – Protein was determined according to Lowry et al. (16). Bovine serum albumin was used as a standard. The absorbance at 280 nm was also measured throughout the purification steps.

Assay of Enzymatic Activities – The enzyme assay for arylamidase was carried out as described previously (10) with a slight modification. In this study, enzyme preparation was previously treated with 0.05 M potassium phosphate buffer, pH 7.0, containing 0.5 mM dithiothreitol at 37°C for 15 min and arylamidase activity was determined using L-alanine β-naphthylamide as a substrate. The enzyme assay for the hydrolysis of various peptides was conducted by the method of Nicholson and Kim (17) with a slight modification. The enzyme, 0.1 ml (0.59 μg) was previously treated with 0.05 M potassium phosphate buffer, pH 7.0, containing 0.5 mM dithiothreitol at 37°C for 15 min. To the enzyme solution, 0.25 ml of various peptides (200 nmoles) previously dissolved in 0.05 M potassium phosphate buffer, pH 7.0 was added. The mixture was incubated at 37°C for 60 min. The reaction was stopped by heating for 5 min in a boiling water. To the mixture, 0.5 ml of 0.05 M potassium phosphate buffer, pH 7.0, containing 100 μg of L-amino acid oxidase, 10 μg of horseradish peroxidase and 50 μg of o-dianisidine were added. The mixture was incubated at 37°C for 20 min. The reaction was stopped by adding 0.3 ml of 50 % sulfuric acid. The absorbance at 530 nm was measured using a Hitachi 124 spectrophotometer. L-Leucine was used as a standard amino acid.

Enzyme Purification – A purification of an arylamidase from five Japanese monkey brains was carried out as described previously (10). All operations

were performed at 0-4°C and the buffer system contained 5 mM β-mercaptoethanol unless otherwise indicated.

Step 1) Five Japanese monkey brains (280 g) were homogenized in a Waring blendor for 3 min with 3 vol of 0.25 M sucrose, 0.01 M potassium phosphate buffer, pH 7.0, and the homogenate was centrifuged at 30,000 x g for 60 min. The supernatant was collected.

Step 2) Solid $(NH_4)_2SO_4$ was added to the extract (660 ml) and the precipitates between 40 and 70 % saturation were collected by centrifugation at 10,000 x g for 30 min. The precipitates were dissolved in 0.01 M potassium phosphate buffer, pH 6.5, and the solution was dialyzed overnight against the same buffer.

Step 3) The dialyzed solution (83 ml) was applied to a hydroxylapatite column (2.6 x 40 cm) previously equilibrated with 0.01 M potassium phosphate buffer, pH 6.5. The adsorbed proteins were eluted with linearly increasing potassium phosphate concentration from 0.01 M to 0.5 M at a rate of flow of 12 ml/h.

Step 4) The fractions containing arylamidase activity from Step 3) were dialyzed against 0.01 M potassium phosphate buffer, pH 7.0. The dialyzed solution (108 ml) was applied to a column of DEAE-cellulose (1.5 x 85 cm) previously equilibrated with 0.01 M potassium phosphate buffer, pH 7.0. The adsorbed proteins were eluted with linearly increasing potassium phosphate concentration from 0.01 M to 0.4 M at a rate of flow of 12 ml/h.

Step 5) The active fractions from Step 4) were concnetrated to a small volume (13.5 ml) by ultrafiltration in a Diaflo MC-2 ultrafiltration cell with a reservoir (Bio Engineering Co.) using a G-05T membrane. The solution was further concentrated by dialysis against 0.01 M potassium phosphate

buffer, pH 7.0, containing 40 % sucrose. The dialyzed solution (4.9 ml) was applied to a column of Sephadex G-200 (2.5 x 135 cm) and eluted with 0.01 M potassium phosphate buffer, pH 7.0 at a rate of flow of 18 ml/h. Step 6) The active fractions from Step 5) were dialyzed against 0.01 M potassium phosphate buffer, pH 6.5. The dialyzed solution was applied to a column of hydroxylapatite (1.2 x 14 cm) previously equilibrated with 0.01 M potassium phosphate buffer, pH 6.5. The adsorbed proteins were eluted with linearly increasing potassium phosphate concentration from 0.01 to 0.4 M at a rate of flow of 18 ml/h. The active fractions were concentrated by dialysis against 0.01 M potassium phosphate buffer, pH 7.0, containing 40 % sucrose and stored at -20°C.

Polyacrylamide Gel Electrophoresis – Polyacrylamide gel electrophoresis was carried out in 7.5 % cross-linked polyacrylamide gel according to the method of Davis (18) and Ornstein (19). The protein was stained with Coomasie brilliant blue.

Amino Acid Analysis – Amino acids were determined with a JEOL-6AH amino acid analyzer according to the procedure of Spackman et al. (20). Samples for analysis were hydrolyzed with 1.0 ml of 6 N HCl at 110°C for 24, 48, and 72 h in evaculated sealed tubes. The content of tryptophan was determined spectrophotometrically by the method of Goodwin and Morton (21).

Analysis of Amino-terminal Residue – Amino-terminal residue of protein was analyzed by the method of Gray (22). To 100 μg of protein, 50 μl of 1 % SDS was added. The mixture was heated in a boiling water for 5 min.

After the solution was cooled, 50 µl of N-ethylmorpholine and 75 µl of dansyl chloride (25 mg/ml of dimethylformamide) were added. After standing for 1 h at room temperature, 500 µl of acetone was added. The precipitated protein was collected by centrifugation. The dansylated amino acid was identified by polyamide layer (5 x 5 cm) according to the procedure of Woods and Wang (23). The spot was detected by ultraviolet light (Manaslu light, wave length, 2536 Å).

Effects of β-Mercaptoethanol and Dithiothreitol – The enzyme preparation, 0.2 ml (4 µg) was dialyzed against 0.01 M potassium phosphate buffer, pH 7.0 for 3 h to remove excess β-mercaptoethanol. The dialyzed enzyme, 0.1 ml (0.1 µg) was incubated with 0.1 ml of 0.2 mM to 2 mM β-mercaptoethanol and dithiothreitol at 37°C for 15 min. The enzyme activity was determined using L-alanine β-naphthylamide as a substrate. The enzyme activity which was not dialyzed was taken as 100 %.

Stability of Enzyme at Various Temperatures – The enzyme, 0.1 ml (0.1 µg) was previously treated at various temperatures for 15 min and aliquots were transferred to ice bath. The residual enzyme activity was determined using L-alanine β-naphthylamide as a substrate. The enzyme activity which was treated at 37°C under the same condition was taken as 100 %.

Effects of Bestatin and Various Peptides – The enzyme, 0.1 ml (0.1 µg) was mixed with 0.1 ml of various compounds previously dissolved in 0.1 M potassium phosphate buffer, pH 7.0. After incubation of the mixture at 37°C for 15 min, the residual enzyme activity was determined using

L-alanine β-naphthylamide as a substrate. The enzyme activity which was treated with 0.1 ml of 0.1 M potassium phsphate buffer, pH 7.0 under the same condition was taken as 100 %. The Ki value for bestatin was determined by the method of Dixon (24).

Reversibility of Inhibitions by Puromycin and Bestatin – The enzyme preparation, 0.3 ml (2.28 μg) was mixed with 0.3 ml of 2 mM puromycin and bestatin previously dissolved in 0.1 M potassium phosphate buffer, pH 7.0. After incubation of the mixture at 37°C for 30 min, each samples (0.4 ml) were placed on a column of Sephadex G-25 (1 x 19.5 cm) and eluted with 0.05 M potassium phosphate buffer, pH 7.0, containing 0.5 mM dithiothreitol. The enzyme activity was determined using L-alanine β-naphthylamide as a substrate. The enzyme activity which was not treated with these compounds was taken as 100 %.

Analysis of Hydrolysis Products of Various Peptides – The enzyme preparation, 10 μl (0.38 μg) which was previously treated with 0.05 M potassium phosphate buffer, pH 7.0, containing 0.5 mM dithiothreitol at 37°C for 15 min was mixed with 10 μl (5 nmoles) of various peptides previously dissolved in 0.1 M potassium phosphate buffer, pH 7.0. After incubation of the mixture at 37°C for 60 min, the enzyme reaction was stopped by heating for 5 min in a boiling water. The mixture was dried in vacuo and dansylation was carried out by mixing of 10 μl of dansyl chloride (1 mg/ml of acetone) and 10 μl of 0.2 M $NaCO_3$, and incubating at 37°C for 30 min. The reaction products were dried in vacuo and dissolved in 5 μl of 95 % ethanol. The solution (0.5 μl) was chromatographed on polyamide layer (5 x 5 cm) according to the method of Woods and Wang (23).

pH Optimum for L-leucyl-glycyl-glycine – The pH optimum of the enzyme for L-leucyl-glycyl-glycine was determined in 0.1 M potassium phosphate buffer, pH 5.5 to 8.5, using 0.59 µg of the enzyme. The enzyme assay was carried out as described above.

Km Determination for Peptides – The Km values for various peptides were determined by the method of Lineweaver and Burk (25). The substrate concentration used in this study ranged from 7.14×10^{-6}M to 7.14×10^{-4}M. All assays were conducted at 37°C for 10 min at pH 7.0. The amount of the enzyme used was 0.59 µg for each assay.

RESULTS

Purification of Enzyme – A purification of monkey brain arylamidase from 30,000 x g-soluble fraction are summarized in Table I. The chromatographic pattern on the second hydroxylapatite column is shown in Fig.1. The arylamidase was eluted at 0.1-0.14 M potassium phosphate concentration and activity of arylamidase and absorbance at 280 nm were closely parallel. In the above method, the enzyme was purified about 475-fold from 30,000 x g-supernatant fraction with a yield of about 16 %. From five monkey brains, 1.35 mg of the enzyme was obtained. The homogeneity of the final preparation was tested by polyacrylamide gel electrophoresis at pH 8.9. Only one major band was observed (Fig.2).

Amino Acid Composition – The amino acid composition of the enzyme is shown in Table II. The total content of aspartic and glutamic acid was 22.4 %, while that of arginine, lysine and histidine was 13.2 %. These results suggest that the enzyme is an acidic protein. The unique characteristic of the enzyme is low content of cystine. Fairly high contents of alanine, valine and leucine are also noted.

Amino-terminal Amino Acid – By the dansylation method, only dansyl (DNS-) alanine was detected on polyamide layer chromatography.

Effects of Sulfhydryl Compounds on Enzyme Activity – The enzyme lost 72 % of original activity by dialysis against 0.01 M potassium phosphate buffer, pH 7.0, in which β-mercaptoethanol was absent. As shown in Fig.3, the

restoration of enzyme activity was observed on addition of β-mercaptoethanol and dithiothreitol. Complete restoration of the enzyme activity occurred with 0.4 mM to 1 mM β-mercaptoethanol. On the other hand, 0.2 mM to 1 mM dithiothreitol caused about 2.5-fold increase of the original enzyme activity. These results show that sulfhydryl compounds activate the enzyme.

Stability of Enzyme at Various Temperatures – As shown in Fig.4, the enzyme lost its activity gradually with increasing temperature. The enzyme was completely inactivated at 60°C. The result shows that the enzyme is fairly unstable to heat treatment.

Effect of Bestatin on Enzyme Activity – The enzyme lost its activity gradually with increasing concentration of bestatin (Fig.5). The extents of inhibition were 86 % and 97 % with 0.1 mM and 1 mM bestatin, respectively. The inhibition was competitive and the Ki value was calculated to be 2.5×10^{-7}M. The enzyme did not hydrolyze bestatin itself. When bestatin was incubated with the enzyme, L-leucine was not detected by polyamide layer chromatography.

Reversibility of Inhibitions by Puromycin and Bestatin – After gel filtration a mixture of the enzyme and each inhibitor, in which the enzyme was completely inhibited, about 90 % of the enzyme activity was restored. The results show that inhibitions by puromycin and bestatin are reversible.

Effects of Various Peptides on Enzyme Activity – Table III shows the inhibitory

effects of various peptides on the enzyme activity. About 60-70 % inhibitions were observed with Lys-bradykinin, angiotensin I and substance P. No inhibition was observed with L-leucyl-glycyl-glycine, L-leucyl-glycine, L-alanyl-alanyl-alanine, L-alanyl-alanine, TRH and LH-RH. L-Phenylalanyl-sarcosine which contains N-methylated peptide link and carbobenzoxy-L-phenylalanyl-tyrosine of which the free amino terminal residue is blocked did not show any appreciable inhibitory effects.

Hydrolysis of Various Peptides – The amounts of amino acids released from various peptides by the enzyme are shown in Table IV. The enzyme hydrolyzed di-, tri- and oligopeptides. The enzyme preferentially hydrolyzed the peptides which possess a neutral amino acid at the N-terminal residue such as enkephalins and Met-Lys-bradykinin. Enkephalins were most rapidly hydrolyzed under the conditions used. The enzyme also hydrolyzed angiotensin I and II of which the N-terminal amino acid is an acidic amino acid (aspartic acid), whereas the enzyme hydrolyzed Lys-bradykinin only slightly. On the other hand, the peptides such as TRH and LH-RH which possess a pyrrolidonecarboxylyl group at the N-terminal position were not hydrolyzed by the enzyme. When synthetic L-pyrrolidonyl β-naphthylamide was used as substrate, no arylamidase activity was observed. The enzyme also did not hydrolyze the peptides such as substance P and bradykinin which possess a proline residue adjacent to the N-terminal residue, while the peptide (MIF) which possesses a proline residue at the N-terminal position was hydrolyzed by the enzyme.

Km Values – The Km values for some peptides were presented in Table IV. The highest Km value was observed for L-leucyl-glycine and the lowest

Km value was observed for Met-Lys-bradykinin. The Km values gradually decrease with increasing the length of the side chain. The results suggest that the enzyme has relatively high affinity toward longer peptides.

Inhibition of Hydrolysis of Peptides by Puromycin and Bestatin – The hydrolysis of peptides by the enzyme was competitively inhibited by puromycin and bestatin. The Ki values for puromycin and bestatin with L-leucyl-glycyl-glycine as a substrate were calculated to be 1.2×10^{-6} and 9×10^{-7}M, respectively. The hydrolysis of various physiologically active peptides was also inhibited by puromycin and bestatin. Pretreatment of the enzyme with 1 mM puromycin or bestatin at 37°C for 15 min resulted in complete loss of the enzyme activity.

pH Optimum for L-leucyl-glycyl-glycine – The effect of pH on the rate of the hydrolysis of L-leucyl-glycyl-glycine is shown in Fig.6. The optimum was observed at pH 7.0. The pH optimum and its shape were almost the same as those for L-alanine β-naphthylamide described in the previous paper (10).

Analysis of Hydrolysis Products of Various Peptides – In order to analyze the hydrolysis products of various peptides by the enzyme, polyamide layer chromatography was employed to detect liberated amino acids as dansylated derivatives. No DNS-amino acids were detected in the cases of LH-RH, TRH, substance P and bradykinin. On the other hand, all DNS-amino acids, bis-DNS-tyrosine, DNS-glycine, DNS-phenylalanine, DNS-leucine and DNS-methionine

were detected in the cases of Leu^5-enkephalin and Met^5-enkephalin. When Lys-bradykinin was used as substrate, only bis-DNS-lysine was found, while both DNS-methionine and bis-DNS-lysine were found in the case of Met-Lys-bradykinin. The DNS-derivatives of the first five amino acids, DNS-aspartic acid, DNS-arginine, DNS-valine, bis-DNS-tyrosine and DNS-isoleucine were detected in the cases of angiotensin I and II. DNS-proline, DNS-leucine and DNS-glycine-NH_2 were found in the case of MIF. The results are summarized in Table V. When synthetic peptides, L-leucyl-glycyl-glycine and L-leucyl-glycine were used as substrate, DNS-leucine and DNS-glycyl-glycine were detected in the case of L-leucyl-glycyl-glycine, and DNS-leucine and DNS-glycine were detected in the case of L-leucyl-glycine. Similarly, L-alanyl-alanyl-alanine and L-alanyl-alanine were used as substrate, DNS-alanine was detected. L-Alanyl-proline was not hydrolyzed by the enzyme under the conditions used. Pretreatment of the enzyme with 1 mM puromycin or bestatin at 37°C for 15 min completely inhibited the release of amino acids from various peptides.

DISCUSSION

Extensive purification of a group c) arylamidase from five monkey brains has been carried out. The final yield and purification factor were relatively in good agreement with the results of the previous work (10). The final preparation appeared to be one band on 7.5 % polyacrylamide gel electrophoresis, pH 8.9 and only one amino acid (alanine) was detected by N-terminal analysis. The result suggests that the enzyme is composed of a single polypeptide chain.

The amino acid composition of the enzyme indicates that the enzyme is fairly rich in acidic amino acids. The total content of aspartic acid and glutamic acid is about 1.7-fold over that of lysine, arginine and histidine. The high contents of acidic amino acids over basic amino acids suggest that the enzyme is an acidic protein. This agrees with the fact that the enzyme was strongly adsorbed to DEAE-cellulose and was eluted with relatively high concentration of potassium phosphate buffer. The significant amounts of alanine, valine and leucine and low content of cystine are also noted in amino acid composition of the enzyme. Recently, human liver aminopeptidase which hydrolyzed L-alanine β-naphthylamide has been purified and its amino acid composition has been reported (26). The high content of acidic amino acids and trace of cystine of human liver enzyme are fairly similar to those of monkey brain arylamidase.

An appreciable loss of the enzyme activity was observed by dialysis against the buffer which did not contain sulfhydryl compound. Complete restoration of the enzyme activity occurred with 0.4 mM to 1 mM β-mercaptoethanol and about 2.5-fold increase of the enzyme activity was observed with 0.1 mM to

1 mM dithiothreitol. These results indicate that sulfhydryl compounds activate the enzyme. Dithiothreitol seems to be more effective than β-mercaptoethanol. These properties of the enzyme coincide with those of rat brain and bovine brain arylamidases (1,2).

The enzyme was fairly unstable on heat treatment. Treatment of the enzyme at 60°C for 15 min resulted in complete loss of the enzyme activity.

Bestatin is now known as a specific inhibitor for aminopeptidase B and leucine aminopeptidase (11-13). The Ki values for aminopeptidase B and leucine aminopeptidase have been reported to be 6×10^{-8} and 2×10^{-8}M, respectively. The results presented in Fig.5 shows that the monkey brain arylamidase was also strongly inhibited by bestatin. The inhibition was competitive and Ki value was calculated to be 2.5×10^{-7}M, which was of the same order as that of puromycin (5×10^{-7}M). The slight differences in Ki values of bestatin for aminopeptidase B, leucine aminopeptidase and monkey brain arylamidase cannot be explained at present, but perhaps reflect the differences in enzyme or in substrate used. A free amino group and CO-NH bond are present in both puromycin and bestatin molecules. Similar inhibitions by some peptides (Table III) suggest that the free amino group and CO-NH bond are perhaps essential for the interaction of the enzyme with puromycin and bestatin.

The results presented in Table IV show that the enzyme hydrolyzes di-, tri- and oligopeptides. These properties are distinct from those of rat brain and bovine brain arylamidases (1,3). These arylamidases have been reported to be inactive toward L-leucyl-glycine and L-leucyl-glycyl-glycine. These differences may reflect differences in species or in assay method used. The method used in this study, L-amino acid oxidase method, is very sensitive

toward L-amino acids and the blank absorbance of peptide is very low (17). Under the conditions used, the enzyme preferentially hydrolyzed the peptides such as enkephalins and Met-Lys-bradykinin which possess a neutral amino acid at the N-terminal positions. The enzyme also hydrolyzed angiotensin I and II of which the N-terminal amino acid is an acidic amino acid (aspartic acid). On the other hand, the enzyme only slightly hydrolyzed Lys-bradykinin. In the previous work, basic amino acid β-naphthylamide (L-arginine β-naphthylamide) was found to be hydrolyzed with a relatively high rate (63 % of the rate of hydrolysis of L-alanine β-naphthylamide). This discrepancy may be due to the conformation in the N-terminal moiety of Lys-bradykinin. More detailed studies will be necessary in this regard. The enzyme did not hydrolyze TRH and LH-RH which possess pyrrolidonecarboxylyl group at the N-terminal positions. In addition, synthetic L-pyrrolidonyl β-naphthylamide was not hydrolyzed by the enzyme. These results suggest that the enzyme cannot release N-terminal pyrrolidonecarboxylyl group. These physiologically active peptides which regulate the release of peptide hormones from adenohypophysial cells may be degraded by other enzymes such as pyrrolidonyl peptidase and a brain neutral endopeptidase reported elsewhere (27,28). MIF which possesses a proline residue at the N-terminal position was hydrolyzed by the enzyme. The result coincides with the results obtained in the previous study, in which L-proline β-naphthylamide was found to be hydrolyzed at a rate of 19 % of that of L-alanine β-naphthylamide. Simmons et al. (29) have described specific aminopeptidases for MIF in bovine brain tissue. These aminopeptidases have been reported to hydrolyze L-leucyl-glycyl-glycine and to be different from arylamidase. In monkey brain tissue,

aminopeptidase which was active toward L-leucyl-glycyl-glycine and was inactive toward arylamidase substrate was also found (Hayashi, M., unpublished result). The purification and characterizations of this aminopeptidase are now in progress. The peptides such as substance P and bradykinin which possess a proline residue adjacent to the N-terminal residue were not hydrolyzed by monkey brain arylamidase. Recently, Marks et al. (30) and Benuck et al. (31) have reported that bradykinin and substance P were degraded by a rat brain neutral endopeptidases. The resistance of these peptides toward the hydrolysis by brain arylamidase suggest that brain arylamidase does not participate in the initial deactivation of these physiologically active peptides. The purification and characterization of these endopeptidases remains to be done in the future.

The results obtained in Km determination indicate that the enzyme has high affinity toward longer peptides. These phenomena may reflect the effects of the C-terminal carboxyl group or high affinity toward naturally-occurring physiologically-active peptides.

The hydrolysis of peptides by the enzyme was also competitively inhibited by puromycin and bestatin. In addition, treatment of the enzyme with 1 mM puromycin or bestatin resulted in complete loss of aminopeptidase activity toward various physiologically active peptides. These results show that these reagents inhibit not only arylamidase activity but also aminopeptidase activity of the enzyme.

The pH optimum for L-leucyl-glycyl-glycine was pH 7.0 which was the same as that for arylamidase substrate.

Studies on the hydrolysis products of various peptides indicate that

the enzyme hydrolyzes peptide substrate sequencially from the N-terminal position. This shows that the enzyme is an exopeptidase. Among various physiologically active peptides, no amino acids were released from TRH, LH-RH, substance P and bradykinin by the enzyme, which confirm the results of aminopeptidase activities toward these peptides (Table IV). Only lysine was released from Lys-bradykinin and methionine and lysine were released from Met-Lys-bradykinin. These results indicate that the monkey brain arylamidase has kinin-converting activity. The partially purified rabbit brain enzyme which shows kinin-converting activity has been reported to have arylamidase activity and has been competitively inhibited by puromycin (9). To clarify the enzymatic identity or nonidentity between monkey brain arylamidase and this rabbit brain enzyme, purification of rabbit brain arylamidase will be necessary. First five amino acids were detected in the hydrolysis products of angiotensin I and II. Abrash et al. (8) have reported similar findings, in which angiotensin II has been degraded by pig brain arylamidase which hydrolyze both basic and neutral amino acid β-naphthylamides. The roles of angiotensin II in the central nervous system that are associated with a pressor response, drinking behavior and release of antidiuretic hormone have been reviewed in the literature (32). The results of the degradation of this peptide by brain arylamidase suggest that the enzyme has an angiotensinase activity and participates in the degradation of the peptide in brain tissues.

All amino acids were detected in the hydrolysis products of Met^5- and Leu^5-enkephalin. Enkephalins are now known as a morphin-like factor or natural peptides that react with opiate receptor (33,34). These peptides have been

found in only vertebrate brains (35) and also found in monkey brain (36,37). In rat brain homogenates and rat and human plasma, the enzymes which release N-terminal tyrosine residue have been described (38,39). These enzymes have not well been characterized yet. The arylamidase activity was found to be fairly evenly distributed among various regions of monkey brain tissues including cerebral gray matter, cerebral white matter, thalamus-hypothalamus, amygdala and cerebellar cortex (Hayashi, M., unpublished result). Activity of enkephalin has been also found in various regions of monkey brain, but the highest activity has been found in the caudate nucleus (36). Although the arylamidase activity in the caudate nucleus of monkey brain was not determined, the results in this study suggest that brain arylamidase may be one of the enzymes participating in the degradation of these peptides.

From the above results, it may be expected that brain arylamidase participates in the release and the degradation of some physiologically active peptides and that application of inhibitors aids in clarifing metabolism of these peptides in brain tissues.

ACKNOWLEDGMENTS

The author wishes to thank Dr. K.Oshima (Department of Physiology) and Dr. K.Takahashi (Department of Biochemistry) for their valuable discussions. I also thank Dr. H.Umezawa and Dr. T.Aoyagi (Institute of Microbial Chemistry, Tokyo) for generous supply of bestatin.

REFERENCES

1. Marks, N., Datta, R.K., & Lajtha, A. (1968) J.Biol.Chem. 243, 2882-2889
2. Brecher, A.S., & Suszkiw, J.B. (1969) Biochem.J. 112, 335-342
3. Suszkiw, J.B., & Brecher, A.S. (1970) Biochemistry 9, 4008-4017
4. Patterson, E.K., Hsiao, S.-H., & Keppel, A. (1963) J.Biol.Chem. 238, 3611-3620
5. Marks, N., & Lajtha, A. (1970) Protein Metabolism of the Nervous System (Lajtha, A., ed.), pp. 39-75 Plenum Press, New York
6. Appel, W. (1974) Method of Enzymatic Analysis (Bergmeyer, H.U., ed.), Vol.2, pp.949-996 Verlog Chemie, Weinheim/Academic Press, New York
7. Hopsu-Havu, V.K., Mäkinen, K.K., & Glenner, G.G. (1966) Nature 212, 1271-1272
8. Abrash, L., Walter, R., & Marks, N. (1971) Experientia 27, 1352-1353
9. Camargo, A.C.M., Ramalho-Pinto, F.J., & Greene, N.J. (1972) J.Neurochem. 19, 37-49
10. Hayashi, M., & Oshima, K. (1977) J.Biochem. 81, 631-639
11. Umezawa, H., Aoyagi, T., Suda, H., Hamada, M., & Takeuchi, T. (1976) J.Antibiotics 24, 97-99
12. Suda, H., Takita, T., Aoyagi, T., & Umezawa, H. (1976) J.Antibiotics 24, 100-102
13. Suda, H., Aoyagi, T., Takeuchi, T., & Umezawa, H. (1976) Arch.Biochem.Biophys. 177, 196-200
14. Hayashi, M., & Oshima K. (1977) Seikagaku (in Japanese) 49, 759
15. Szeczuk, A., & Mulczyk, M. (1969) Eur.J.Biochem. 8, 63-67
16. Lowry, O.H., Rosbrough, N.J., Farr, A.L., & Randall, R.J. (1951) J.Biol.Chem. 193, 265-275

17. Nicholson, J.A., & Kim, Y.S. (1975) Anal.Biochem. 63, 110-117

18. Davis, B.J. (1964) Ann.N.Y.Acad.Sci. 121, 404-427

19. Ornstein, L. (1964) Ann.N.Y.Acad.Sci. 121, 321-349

20. Spackman, D.H., Stein, W.H., & Moore, S. (1956) Anal.Chem. 30, 1190-1206

21. Goodwin, T.W. & Morton, R.A. (1946) Biochem.J. 40, 628-632

22. Gray, W.R. (1972) Methods in Enzymology (Hirs, C.H.W., & Timasheff, S.N., eds.) Vol.XXV, pp.121-138 Academic Press, New York

23. Woods, K.R., & Wang, K.T. (1967) Biochim.Biophys.Acta 133, 369-370

24. Dixon, M. (1953) Biochem.J. 55, 170-171

25. Lineweaver, H., & Burk, D. (1934) J.Amer.Chem.Soc. 56, 658-666

26. Starnes, W.L., & Behal, F.J. (1974) Biochemistry 13, 3221-3227

27. Szewczuk, A., & Kwiatkowska, J. (1970) Eur.J.Biochem. 15, 92-96

28. Marks, N., & Stern, F. (1974) Biochem.Biophys.Res.Commun. 61, 1458-1463

29. Simmons, W.H., & Brecher, A.S. (1973) J.Biol.Chem. 248, 5780-5784

30. Marks, N., & Pirotta, M. (1971) Brain Research 33, 565-567

31. Benuck, M., & Marks, N. (1975) Biochem.Biophys.Res.Commun. 65, 153-160

32. Barker, J.L. (1976) Physiological Reviews 56, 435-452

33. Hughes, J. (1975) Brain Research 88, 295-308

34. Hughes, J., Smith, T., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A., & Morris, H.R. (1975) Nature 258, 577-579

36. Simantov, R., Kuhar, M.J., Pasternak, G.W., & Snyder, S.H. (1976) Brain Research 106, 189-197

37. Kuhar, M.J., Pert, C.B., & Snyder, S.H. (1975) Nature 262, 782-783

38. Hambrook, J.M., Morgan, B.A., Rance, M.J., & Smith, C.F.C. (1976) Nature 262, 782-783

39. Marks, N., Grynbaum. A., & Neidle, A. (1977) Biochem.Biophys.Res.Commun. 74, 1552-1559

TABLE I. Summary of the purification for arylamidase from monkey brain.

Fraction	Total protein (mg)	Total activity (units x 10^{-2})	Specific activity (units/mg)	Yield (%)	Purification factor
30,000 x g supernatant	4,066	823.0	20.2	100	1
Precipitates at 40-70% $(NH_4)_2SO_4$	1,829	727.7	39.8	88	1.97
First hydroxylapatite	80.8	449.4	556.2	55	27.5
DEAE-cellulose	12.4	312.6	2521	38	125
Sephadex G-200	4.8	205.2	4275	25	212
Second hydroxylapatite	1.35	129.5	9593	16	475

TABLE II. Amino acid composition of monkey brain arylamidase[a]

Amino acid	Number of residue per molecule of protein
Lys	53.7 (54)
His	18.6 (19)
Arg	36.6 (37)
Asp	83.0 (83)
Thr	39.4[b] (39)
Ser	46.2[b] (46)
Glu	104.1 (104)
Pro	42.1 (42)
Gly	56.0 (56)
Ala	67.9 (68)
Cys/2	7.4 (7)
Val	64.5 (65)
Met	15.4 (15)
Ile	39.4 (39)
Leu	83.8 (84)
Tyr	26.6 (27)
Phe	37.2 (37)
Trp	12.7[c] (13)
Total	835

[a] The values were calculated assuming the molecular weight of arylamidase to be 92,000. Except for threonine, serine, and tryptophan, each value is an average of values obtained for three different periods of hydrolysis. The values in parenthese are nearest integers. [b] Values extrapolated to zero time of hydrolysis. [c] Determined by the method of Goodwin and Morton (21).

TABLE III. Effect of Various peptides on hydrolysis of L-alanine β-naphthylamide by the enzyme. The enzyme (0.1 μg) was previously incubated with various peptides at 37°C for 15 min and residual enzyme activity was determined using L-alanine β-naphthylamide as a substrate.

Peptides (0.25 mM)	Inhibition (%)
Lys-Bradykinin	76
Angiotensin I	70
Substance P	60
Bradykinin	48
Met^5-Enkephalin	40
Leu^5-Enkephalin	14
L-phenylalanyl-sarcosine	5
carbobenzoxy-L-phenylalanyl-tyrosine	0
L-leucyl-glycyl-glycine, L-leucyl-glycine, L-alanyl-alanyl-alanine, L-alanyl-alanine, LH-RH, TRH	0

TABLE IV. Amounts of amino acids released from various peptides by the enzyme and Km values for peptides. The reactions were carried out under the conditions described in the Experimental section using 0.59 μg of the enzyme. L-Amino acid oxidase assay was employed for the measurement of L-amino acids. L-Leucine was used as a standard amino acid.

Substrate	Leucine equivalent (nmoles)	Km (μM)
L-leucyl-glycine	19	625
L-leucyl-glycyl-glycine	115	217
Met^5-Enkephalin	137	167
Leu^5-Enkephalin	130	161
Met-Lys-Bradykinin	104	25
MIF	29	–
Angiotensin I	24	–
Angiotensin II	23	42
Lys-Bradykinin	1	–
TRH, LH-RH, Substance P, Bradykinin	0	–

TABLE V. Summary of hydrolysis of physiologically active peptides by monkey brain arylamidase. Arrows indicate the peptide bonds that are cleaved by the enzyme.

	Substrate and hydrolyzed site
Met^5-Enkephalin	Tyr-Gly-Gly-Phe-Met
Leu^5-Enkephalin	Tyr-Gly-Gly-Phe-Leu
Angiotensin I	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
Angiotensin II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
MIF	Pro-Leu-Gly-NH_2
Met-Lys-Bradykinin	Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Lys-Bradykinin	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

Legend of Figure

Fig.1 Chromatography of monkey brain arylamidase on a second hydroxylapatite column. The enzyme fraction obtained from the Sephadex G-200 column was applied to a column of hydroxylapatite (1.2 x 14 cm). The adsorbed proteins were eluted increasing the concentration of phosphate linearly from 0.01 to 0.4 M (pH 6.5). Each fraction was 5 ml.
○, Absorbance at 280 nm; ●, enzyme activity; —·—, phosphate concentration.

Fig.2 Disc electrophoresis of the purified enzyme. The final enzyme preparation (6.5 μg) was placed on a column of 7.5 % polyacrylamide gel, pH 8.9. The protein was stained with Coomassie brilliand blue. The arrow indicates the position of Bromophenol blue.

Fig.3 Effects of sulfhydryl compounds on the activity of the enzyme. Enzyme preparation which was not dialyzed was taken as 100 % active.
●, β-Mercaptoethanol; ○, dithiothreitol.

Fig.4 Effects of temperature on the activity of the enzyme. Enzyme which had been treated at 37°C for 15 min was taken as 100 % active.

Fig.5 A) Effect of bestatin on the activity of the enzyme. Enzyme which had not been treated with bestatin was taken as 100 % active.
B) Inhibition of the enzyme activity by bestatin. The Ki value was determined by the method of Dixon (24). The concentration of L-alanine β-naphthylamide were 0.25 mM (●), 0.1 mM (○), and 0.05 mM (△).

Fig.6 Effect of pH on the hydrolysis of L-leucyl-glycyl-glycine by the enzyme. The enzyme activity was measured in 0.1 M potassium phosphate buffer, using 0.59 μg of the enzyme. The maximal activity was taken as 100 %.

Fig 1

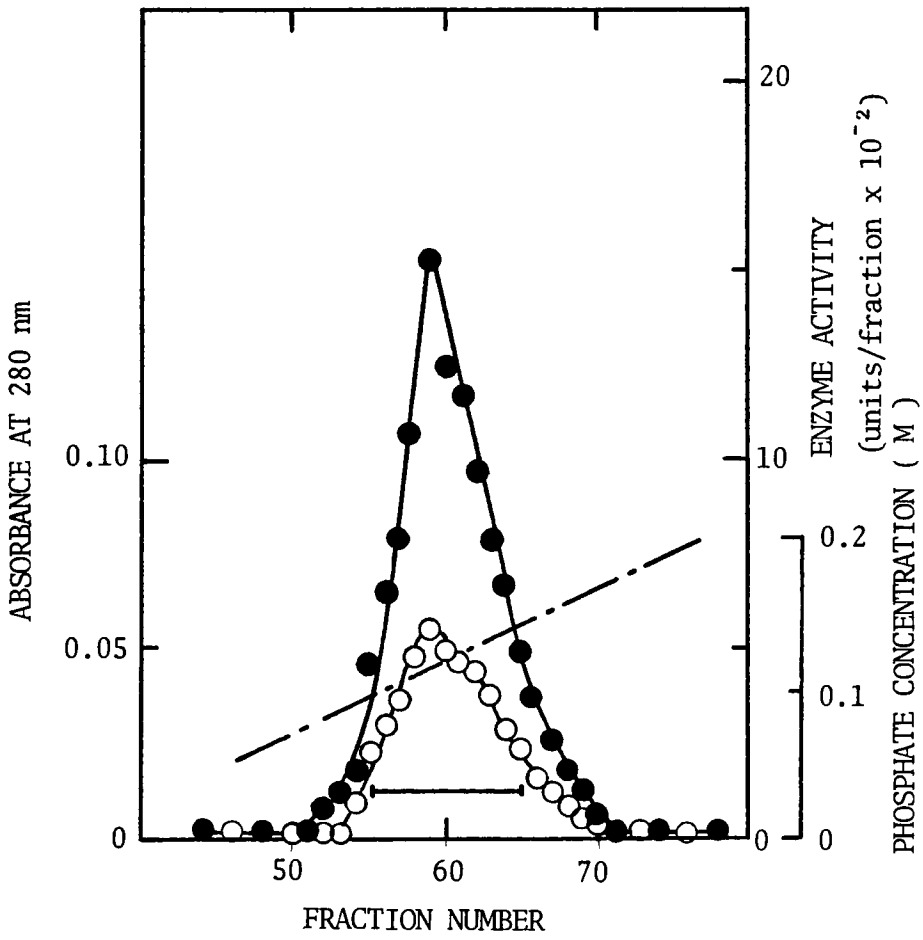
ABSORBANCE AT 280 nm
0.10
0.05
0
50
60
70
FRACTION NUMBER
20
10
0
ENZYME ACTIVITY (units/fraction x 10^{-2})
0.2
0.1
0
PHOSPHATE CONCENTRATION (M)

Fig 2

Fig 3

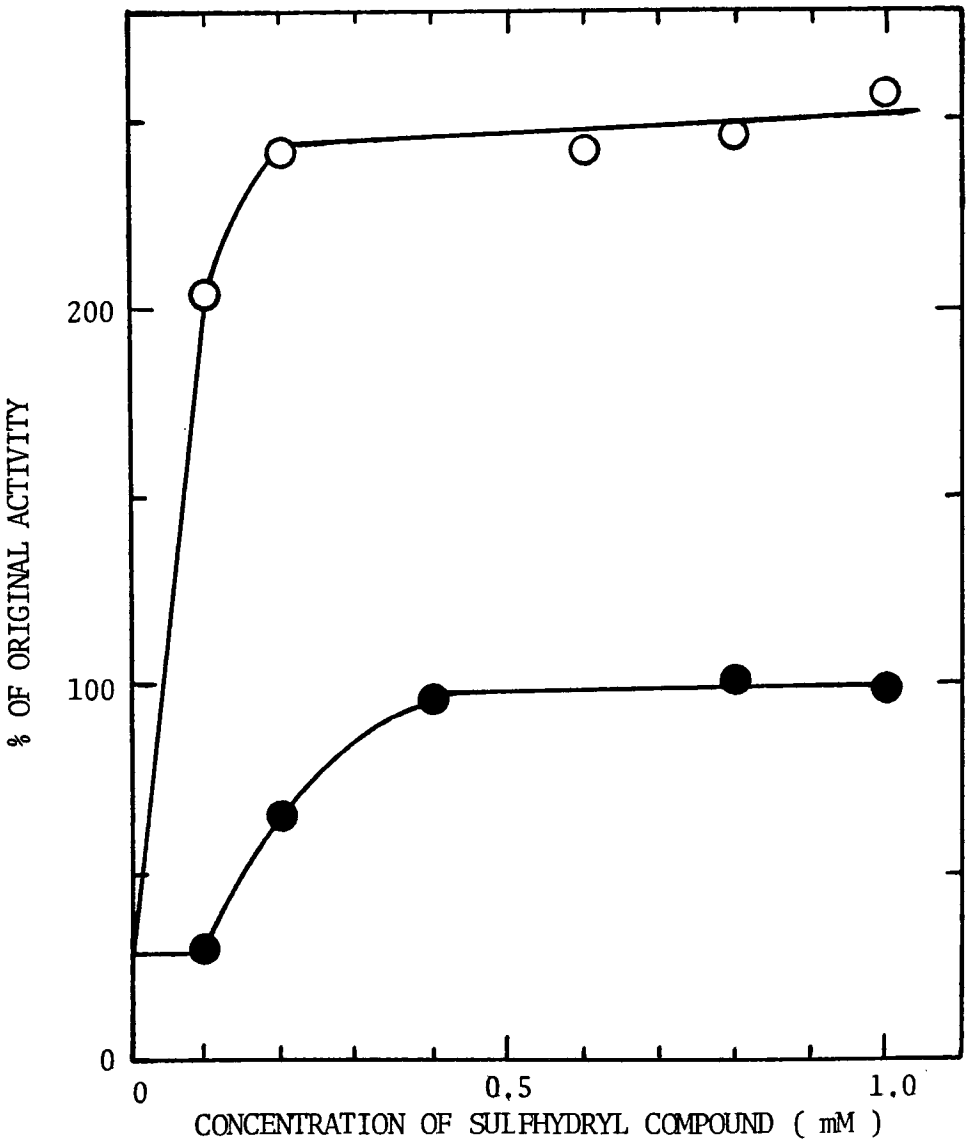
% OF ORIGINAL ACTIVITY
200
100
0
0
0.5
1.0
CONCENTRATION OF SULPHYDRYL COMPOUND (mM)

Fig 4

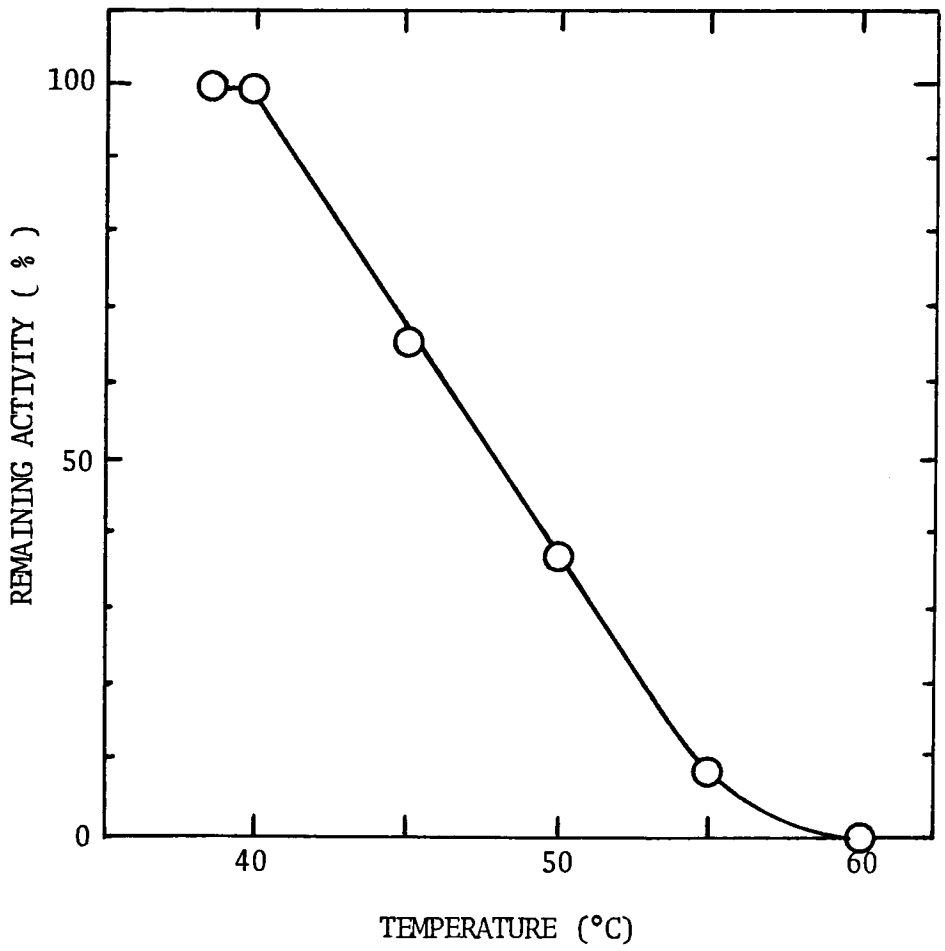

Fig 5

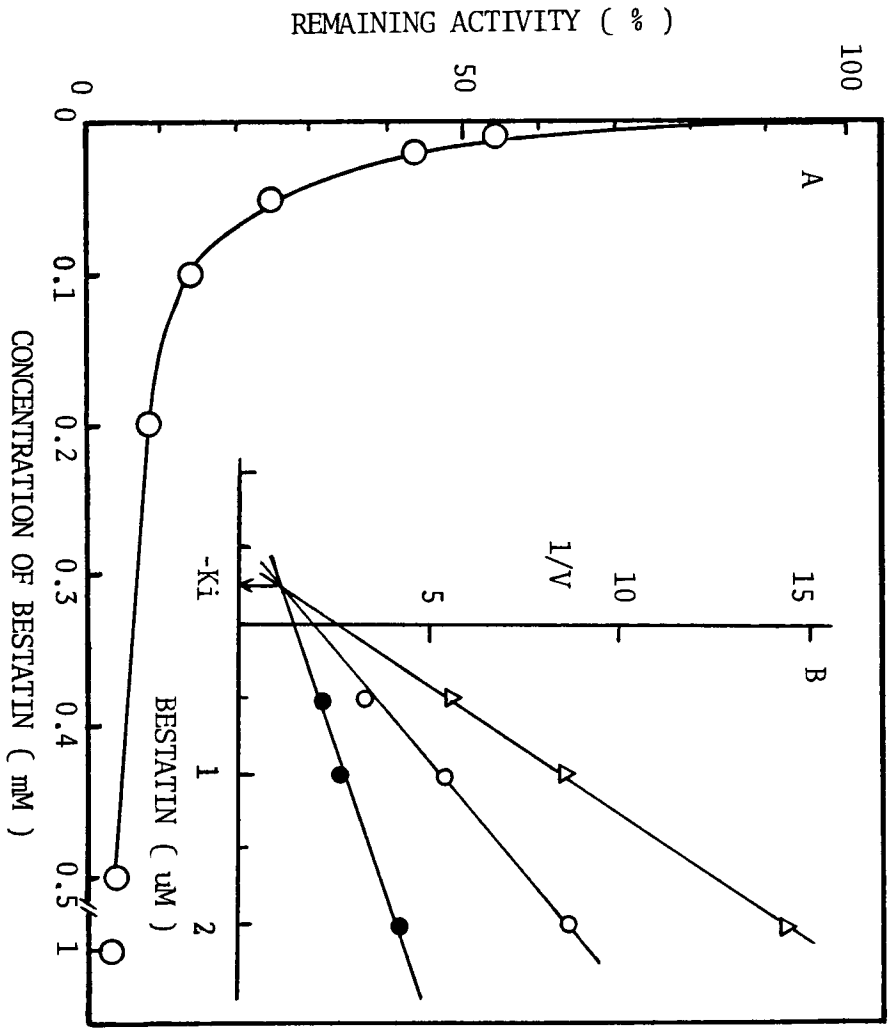
REMAINING ACTIVITY (%)
100
50
0
0
0.1
0.2
0.3
0.4
0.5
1
CONCENTRATION OF BESTATIN (mM)
A
B
1/V
5
10
15
-Ki
1
2
BESTATIN (uM)

Fig 6